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Differential Ability of Surface and Endosomal TLRs To Induce CD8 T Cell Responses In Vivo

Rajakumar Mandraju,1 Sean Murray,1 James Forman, and Chandrashekhar Pasare

TLR activation on dendritic cells (DCs) induces DC maturation and secretion of proinflammatory cytokines, both of which are important for activation and differentiation of CD4 T cells. The importance of TLR activation on DCs for CD8 T cell responses is less clear. In this study, we tested the ability of different TLRs to regulate CD8 T cell responses to pathogens. We found that although all TLRs are able to induce CD8 T cell activation in vitro, there are profound differences in their ability to activate CD8 T cells in vivo. The nucleic acid–recognizing endosomal TLRs, TLR3 and TLR9, had a potent ability to induce CD8 T cell activation. However, the surface TLRs, TLR2 and TLR4, that recognize bacterial ligands were not only incapable of inducing CD8 T cell priming, but they had a dominant effect of inhibiting CD8 T cell expansion induced by activation of endosomal TLRs. We found that TLR2 and TLR4, acting in a MyD88-dependent manner, influenced CD8 T cell priming by altering the composition of DCs in the draining lymph nodes. Our results have important implications for combined bacterial and viral infections and suggest that bacterial infections could constrain the ability of the host to mount effective antiviral CD8 T cell immunity. The Journal of Immunology, 2014, 192: 000–000.

The presentation of pathogen-derived peptides by MHC class II or class I molecules, either by classical or cross-presentation pathways, all achieve the activation of the adaptive immune response. The primary sensing of pathogens by dendritic cells (DCs) is, however, accomplished by pattern recognition receptors (PRRs) that not only induce activation of DCs, but also regulate the trafficking of cargo to maximize peptide generation. Many different PRRs, such as TLRs, retinoic acid–inducible gene I–like receptors (NLRs), and C-type lectin receptors (6), have all been implicated in inducing DC maturation and regulating adaptive immunity. TLRs recognize conserved microbial products from a diverse class of pathogens and initiate signaling to induce inflammatory responses. The outcome of signaling is determined by specificity of the adapter usage by each TLR (7). TLRs use MyD88 and Toll/IL-1R domain–containing adapter inducing IFN-β (TRIF) to activate NF-κB, MAPKs, and IFN regulatory factors (IRFs) and use BCAP to activate PI3K (9, 10). The capacity of TLRs to activate adaptive immune responses is also determined by the nature of signaling events induced in DCs by different TLRs. For example, the TRIF-dependent pathway of TLR4 signaling is sufficient to induce DC maturation but is not sufficient to induce proinflammatory cytokine production and thus fails to activate measurable CD4 T cell responses (11, 12). All known TLRs except TLR3 signal through MyD88 and induce upregulation of both MHC class I and MHC class II molecules. Whereas CD4 T cell activation is a direct outcome of Ag presentation by mature DCs, the ability of TLR-activated DCs to polarize and prime naive CD4 T cells has been well documented (3, 12–14), the role of different TLRs in the regulation of CD8 T cell responses is less well characterized. TLR9 and TLR3 are found in the endosomal compartment of DCs and can thus encounter intracellular pathogens such as viruses (8). As a result, viral nucleic acids activate these TLRs, allowing for the generation of CD8 T cell responses. TLR9 and TLR3 ligands are also known to induce CD8 T cell responses to soluble protein Ags by enhancing APC cross-presentation (15–19). Immunostimulatory CpG DNA motifs can be found in viral and bacterial genomes, and synthetic CpG DNA has been widely used as an adjuvant to enhance CD8 T cell responses in different experimental models (18, 19). Recent reports have shown that TLR7 ligands increase CD8 T cell responses by enabling the cross-priming ability of different DC subsets via type I IFN production (20).

The role of plasma membrane TLRs, TLR2 and TLR4, in inducing CD8 T cell responses is not entirely clear. It has been proposed that LPS enhances cross-presentation due to the ability of TLR4 to recruit TAP to the endoplasmic reticulum (21). Other studies have also implicated both TLR2 and TLR4 in promoting CD8 T cell priming (22, 23). Despite this, it has been reported that pretreatment of animals with TLR ligands reduces the ability of mice to mount CD8 T cell responses in vivo (24). For example, a recent study has suggested that peptidoglycan contamination of LPS activated NOD receptors, which suppressed cross-presentation of Ags in vitro (25). Another study has implicated LPS in induction of IL-10–producing CD4 T cells that can suppress CD8 T cell responses in vivo (26). The precise mechanisms and general rules of how different TLRs regulate cross-presentation and CD8 T cell priming remain unresolved.
In this study, we examined how surface and endosomal TLRs differentially regulate CD8 T cell responses. Although all TLR ligands induced and enhanced cross-presentation and priming of CD8 T cells in vitro, our results clearly show that only activation of endosomal TLRs enhanced CD8 T cell responses in vivo. In contrast, activation of surface TLRs led to the suppression of CD8 T cell responses induced by endosomal TLR agonists and intracellular pathogens. This study brings to the fore the differential role of surface and endosomal TLRs in Ag cross-presentation by DCs, and it provides critical insights into the importance of engaging the correct TLR to induce productive CD8 T cell responses in vivo.

Materials and Methods

**Mice**

All C57BL/6 (B6) mice used in experiments were 6–12 wk of age and purchased from the University of Texas Southwestern Medical Center Mouse Breeding Core. OT-I RAG−/− mice were purchased from Taconic. GFP B6 mice were purchased from The Jackson Laboratory and bred with OT-I RAG−/− mice to generate GFP OT-I RAG−/− mice. Unless otherwise indicated, all transfer experiments were done using CD8 T cells from OT-I–GFP transgenic (Tg) mice. TRIF knockout (KO), MyD88 KO, IRF3 KO, and CD11c–MyD88 Tg mice (on a MyD88-deficient background) indicated, all transfer experiments were done using CD8 T cells from OT-I RAG−/−GFP B6 mice were purchased from The Jackson Laboratory and bred with OT-I–GFP transgenic (Tg) mice. TRIF knockout (KO), MyD88 KO, IRF3 KO, and CD11c–MyD88 Tg mice (on a MyD88-deficient background) were bred and maintained at the University of Texas Southwestern Medical Center animal facility. All mouse experiments were done as per protocols approved by the University of Texas Southwestern Medical Center’s Institutional Animal Care and Use Committee.

**Antibodies**

Abs were obtained from the following sources: BD Biosciences, CD62L-allophycocyanin, CD25-PE-Cy7, CD4-PE-Cy5, CD25-bio, Ly6G-FTTC, B220-PerCP; BioLegend, CD16/32 purified, CD8α-allophycocyanin/Cy7, CD11c-PE-Cy7, CD11c-allophycocyanin, CD45.2-Pacific Blue, B220-bio, NK1.1-bio, CD11b-bio, CD4-bio, CD16/32-bio, Ly6C-bio, CD11c-PE-Cy7, CD11c-allophycocyanin, CD45.2-Pacific Blue, B220-bio, NK1.1-bio, CD11b-bio, CD4-bio, CD16/32-bio, Ly6C-bio, streptavidin-PE; eBioscience, CD4-AF750, CD11b–Alexa Fluor 700, MHc class II–Pacific Blue, Ly6C-allophycocyanin, F4/80–Alexa Fluor 750; Covance, CD4 (GK1.5); Life Technologies, Qdot 605 streptavidin conjugate; Baylor Tetramer Facility, K5–SINFEKL tetramer–PE; Bio-XCell, anti-Ly6G (clone 1A8) Ab.

**Emulsion preparations**

All emulsions were prepared with 50% of the volume being IFA (Sigma-Aldrich) and the remaining 50% of volume being reagents to be tested using a MicroTip 1000 particles/footpad. Vesicular stomatitis virus (VSV)–ΔSpi-1 mutant was used. LPS from *Escherichia coli* 055:B5 (Sigma-Aldrich), 5 μg/footpad; bacterial lipopolysaccharide (LPS; Pam3CSK4; InvivoGen), 20 μg/footpad; polyinosinic-polycytidylic acid (poly(I:C); Fisher Scientific), 20 μg/footpad; and OVA–Alexa Fluor 647 conjugate (Life Technologies), 20 μg/footpad.

**Virus and bacteria preparations**

*Lysteria monocytogenes* 10403 serotype 1, expressing full-length OVA protein (LM-OVA), was provided by Dr. Hao Shen (University of Pennsylvania School of Medicine, Philadelphia, PA) and was passed on 2 × 10⁶ CFU/footpad. *Salmonella typhimurium* SL1344 ΔSpi-1 mutant was provided by Dr. Denise Monack (Stanford University) and used at 500 CFU/footpad. Heat-killed *S. typhimurium* SL1344 ΔSpi-1 mutant was used at 2.5–5 × 10⁶ particles/footpad. Vascular stomatitis virus (VSV)–expressing full-length OVA protein (VSV-OVA) was provided by Dr. Leo Lefrançois (University of Connecticut Health Center, Farmington, CT) and was used at 10⁶ PFU/footpad.

**In vivo priming using OVA or pathogens with or without TLR ligands**

Cells were isolated and pooled from the spleen and multiple lymph nodes (LNs) of GFP OT-I RAG−/− mice, and CD8 T cells were purified using FACS sorting. A cell suspension was prepared and diluted to a concentration of 10⁶ cells/ml. Mice received 100 μl (~1000 cells) via lateral tail vein injection. The next day, Ag emulsions were prepared and injected into the footpads. Inguinal and popliteal LNs were harvested after 7 d to test for a primary response. When pathogens were used, spleen cells were also collected.

In vivo depletion of regulatory T cells, CD4 T cells, and Ly6G+ cells

In vivo depletion of regulatory T cells (Tregs) was achieved by injecting 100 μg purified anti-CD25 mAb in the lateral tail veins of mice 3 d prior to immunization. Total CD4 T cell depletion resulted from 3 i.p. injections of 200 μg purified anti-GK1.5 mAb on days −4, −1, and 3 of immunization. To deplete Ly6G+ cells, one injection of 500 μg 1A8 mAb was given i.p. 1 d prior to immunization, and another injection was given i.p. on day 4 of postimmunization. Treg and CD4 T cell depletion was confirmed by staining PBMCs for CD4 and CD25. Ly6G+ cell depletion was confirmed by sacrificing a cohort of mice and staining LN cells for Ly6G and Ly6C populations.

Flow cytometry

All samples were run on an LSR II (BD Biosciences). All data were analyzed using FlowJo software (Tree Star).

**Preparation of DCs**

After 18–24 h postimmunization, draining LNs were harvested, pooled together, and processed. When Alexa Fluor 647–tagged OVA (OVA-AF647) was used as the Ag, OVA-AF647+ cells were sorted and collected using a MoFlo sorter (Cytometry). In some experiments, splenic DCs from Flt3-treated B6 mice were used as APCs (27).

**In vivo activation of OT-I CD8 T cells**

LN and spleen were collected from one to three OT-I RAG−/− mice and processed together and sorted to obtain pure, naive CD8 T cells (>99% purity). The sorted OT-I cells were CFSE labeled and cultured with CD11c+ splenic DCs at a ratio of 10:1. OVA was added to the culture at a concentration of 20 μg/ml with or without Pam3CSK4 (200 ng/ml), CpG (1 μM), LPS (100 ng/ml), or poly(I:C) (1 μg/ml). Cells were harvested 3 d later, and stained, and samples were run on the LSR II. Experiments using CFSE-labeled OT-I cells and ex vivo sorted APCs were cocultured at a 5:1 ratio and after 3 d were analyzed by FACS. In some experiments, OT-I cells were cocultured with ex vivo–sorted APCs in a 96-well U-bottom dish. The OT-I cells were held constant at 5 × 10⁶ per well and the APCs were serially diluted. After 3 d the culture was pulsed with [3H]thymidine for 12–16 h and then harvested and subjected to scintillation counting using a MicroBeta TriLux counter (PerkinElmer).

**Quantification of *L.* monocytogenes bacterial burden**

After day 3 and day 7 of infection, draining LNs and/or spleen and livers were harvested. Samples were homogenized in equal volume of water and 50 μl sample was spread onto brain–heart infusion plus streptomycin plates. Plates were incubated at 37°C and colonies were counted the next day. Total CFU were calculated by multiplying the number of colonies per plate with the dilution factor.

**Statistical analysis**

Data are presented as means ± SEM. Statistical analyses were performed using Prism (GraphPad Software), and *p* values were obtained by using two-tailed unpaired Student *t* tests.

**Results**

**Differential regulation of CD8 T cell responses by TLRs**

In the present study we examined the role of surface versus cytosolic TLRs in regulation of CD8 T cell responses both in vitro and in vivo. We used OVA as a model Ag and measured expansion of OT-I CD8 T cells, Tg CD8 T cells that express a TCR specific to the OVA–derived peptide (SINFEKL), as a readout for cross-presentation. To understand the ability of different TLRs to induced cross-presentation, we purified splenic DCs from B6 mice and cocultured them with OT-I cells in the presence of titrating doses of OVA, with or without different TLR ligands. Soluble OVA by itself induced moderate CD8 T cell proliferation as reported before (28–30). However, LPS, BLP (Pam3CSK4), CpG, and poly(I:C) all enhanced the ability of splenic DCs to induce expansion of OT-I CD8 T cells (Supplemental Fig. 1A). These data suggested that there is no inherent difference in the ability between different TLRs to activate CD8 T cells. It is understandable for an endosomal TLR such as...
TLR9 that recognizes viral-derived DNA to induce CD8 T cell priming; however, the benefit of priming CD8 T cells following extracellular bacterial (LPS) recognition by TLR4 is not apparent. We were therefore very interested in pursuing this question further to understand whether both surface and endosomal TLRs have an equivalent ability to induce CD8 T cell priming in vivo.

B6 mice received GFP–OT-I cells and 24 h later were immunized s.c. with OVA in the presence or absence of TLR ligands emulsified in IFA. Immunization with OVA alone induced measurable expansion of OT-I cells in the draining LNs. Immunization with OVA together with endosomal TLR ligands poly(I:C) (TLR3 ligand) or CpG (TLR9 ligand) induced significantly enhanced CD8 T cell responses (Fig. 1A, 1B). This is important because both of these TLRs recognize viral ligands and there is need for the immune system to activate CD8 T cells in such a scenario. However, in complete contrast to the in vitro data, immunization of mice with OVA together with surface TLR ligands, BLP (Pam3CSK4, TLR1/2 ligand), or LPS (TLR4 ligand) led to suppression of OT-I CD8 T cell responses. This was in contrast to the ability of LPS to enhance CD4 T cell activation in vivo (Supplemental Fig. 1B, 1C). Because CpG enhanced CD8 T cell responses significantly (Fig. 1A, 1B), we tested whether LPS and BLP would suppress CpG-induced CD8 T cell priming. The suppression of CD8 T cell expansion was evident even when BLP or LPS was combined with OVA plus cytosolic TLR ligands (Fig. 1C, 1D), suggesting that suppression of CD8 responses by surface TLRs is dominant over the ability of endosomal TLRs to prime CD8 T cells. This suppression was also evident when we measured endogenous CD8 T cell responses, without any OT-I T cell transfer, using the K\(^{b}\)-SIINFEKL tetramer (Fig. 1E, 1F). It is possible that activation of TLR2 or TLR4 induces early activation of CD8 T cells that are then exhausted at later time points. To test this possibility, we conducted a temporal investigation of CD8 T cell expansion at days 5, 7, and 9 following immunization and found that LPS inhibited CD8 T cell responses during all time points (Supplemental Fig. 1D). CD8 T cell expansion was not measurable at time points earlier than day 5 in any of the groups. These data suggest that unlike in vitro priming, in vivo activation of CD8 T cells is a complex process, and surface and endosomal TLRs have diametrically opposite effects on the outcome of CD8 T cell priming. These results prompted us to further dissect the role of different TLR signaling components in regulating priming and expansion of CD8 T cells in vivo.

LPS-mediated CD8 T cell suppression is dependent on TLR4 and is induced by the MyD88-dependent signaling pathway in DCs

LPS is a cell wall component of Gram-negative bacteria, and it is possible that even highly purified commercial-grade LPS preparations can contain other contaminants such as NOD ligands. It has been recently reported that suppression of CD8 T cell activation in vitro is mediated by a contaminant present in LPS, which signals through a cytosolic NOD-like receptor (25). It is not clear whether LPS acts on DCs or CD8 T cells directly to suppress their expansion. We designed our next set of experiments to address both of these issues. We transferred wild-type (WT) OT-I CD8 T cells into TLR2/4 double KO mice and immunized them as described before, either using OVA or OVA mixed with LPS in IFA. The LPS-mediated CD8 T cell suppression was completely abrogated in TLR2/4 double KO mice (Fig. 2A, 2B), suggesting that LPS acts directly via TLR4 and that the in vivo suppression of CD8 T cell responses is not due to contaminating NOD ligands. Furthermore, the data also establish that LPS does not act directly on CD8 T cells and that TLR4 expression in non–T cell compartments, potentially in DCs, could play a major role in LPS-mediated suppression of CD8 T cell responses.

TLRs use several signaling adapters, and specificity of signaling is determined by differential usage of adapter proteins. For example, TLR9 uses MyD88 as its only signaling adapter, and all signaling downstream of TLR9 is abrogated in the absence of MyD88. Not surprisingly, the TLR9 ligand CpG-induced enhancement of cross-priming of CD8 T cells is dependent on MyD88 (data not shown). TLR4 signaling is more complex, as it can signal in response to LPS in the absence of MyD88. The MyD88-dependent signaling pathway of TLR4 activates NF-κB and MAPKs, whereas the MyD88-independent pathway uses TRIF as an adapter protein and, in addition to NF-κB and MAPKs, activates IRF3 (31). We were interested in determining the role of MyD88 and TRIF signaling pathways downstream of TLR4 in influencing CD8 T cell priming in vivo. To do so, we immunized WT, MyD88 KO, and TRIF KO mice with OVA in the presence or absence of LPS. Consistent with previous experiments, OVA plus LPS–immunized WT mice had a reduced CD8 T cell response when compared with OVA alone, whereas OVA plus LPS–immunized MyD88 KO mice showed no impairment/reduction in CD8 T cell responses (Fig. 2C, 2D). TRIF KO mice, however, exhibited a reduced CD8 T cell response to OVA alone compared with WT mice (Fig. 2C, 2D), and this might be a result of compromised basal type I IFN production and signaling in these mice, as type I IFNs are known to play a critical role in priming of CD8 T cells (32, 33). This observation is further supported by experiments in IRF3 KO mice where CD8 T cell priming by OVA alone was reduced compared with WT mice (Supplemental Fig. 2A). The reduced CD8 T cell responses to OVA in TRIF KO mice are still however suppressed by LPS (Fig. 2C), suggesting that LPS-mediated CD8 T cell suppression is induced by signaling pathways downstream of MyD88.

The experiments using TLR2/4 double KO mice suggest that a non–T cell compartment was responsible for TLR4-induced suppression of CD8 responses. Because many myeloid and stromal cells express TLR4, we wanted to narrow down the cell type responsible for suppression of CD8 T cell priming. We have previously generated a chimeric mouse that expresses MyD88 under a CD11c promoter (CD11c-MyD88 Tg), and only CD11c-expressing cells (DCs and macrophages) get activated in response to TLR ligands (34). Because the data above suggest that MyD88 signaling downstream of TLRs is responsible for impairment of CD8 T cell responses, we asked whether MyD88 in DCs is directly responsible for this effect. We immunized WT and CD11c-MyD88 Tg mice with OVA plus CpG or OVA plus CpG plus LPS and measured OT-I expansion as described before. CpG induced expansion of OT-I T cells in both B6 and CD11c-MyD88 Tg mice whereas LPS compromised the ability of CpG to induce expansion of CD8 T cells (Fig. 2E). In the CD11c-MyD88 Tg mice, OVA-induced expansion of CD8 T cells was also inhibited when OVA was coinjected along with LPS (Supplemental Fig. 2B). These data suggest that MyD88, downstream of TLR4 in CD11c-expressing cells, is responsible for inhibition of CD8 T cell activation and expansion in vivo.

TLR2 and TLR4 activation suppresses pathogen-induced CD8 T cell responses in vivo

Viral and bacterial coinfections are common occurrences and can lead to severe morbidity and mortality due to dysregulation of various immune responses. Several reports have shown that polymicrobial sepsis leads to impairment in APC functions and subsequent T cell responses. To examine whether TLR2 and TLR4 activation regulate the outcome of CD8 T cell responses to viral infection, we used VSV-OVA to induce CD8 T cell priming in vivo.
and investigated the ability of LPS and BLP to influence these responses. Mice were infected with live VSV-OVA in the presence or absence of LPS or BLP. VSV-OVA alone elicited strong CD8 T cell responses, both in the draining LNs and the spleen (Fig. 3A).

FIGURE 1. Differential regulation of in vivo CD8 T cell responses by endosomal and plasma membrane TLRs. Mice that received GFP-OT-I T cells were immunized as indicated and cells from the draining LNs were stained using K\textsuperscript{b}-SIINFEKL tetramer. (A) CD8\textsuperscript{+} T cells that express GFP and stain for the class I tetramer are shown. (B) Means ± SEM of quantification of GFP\textsuperscript{+} OT-I T cells as a percentage of total CD8 T cells from three independent mice immunized with OVA and different TLR ligands. (C) Representative plots of OT-I T cell expansion in draining LNs 7 d after immunization. (D) Means ± SEM from three independent mice. (E) Mice without any OT-I T cell transfer were immunized as indicated and cells from draining LNs were stained on day 7 for CD8 and K\textsuperscript{b} tetramer to reveal SIINFEKL-specific CD8 T cell expansion. (F) Means ± SEM of tetramer\textsuperscript{+} CD8 T cells from five independent mice. The data of all of the above experiments are representative of at least three independent experiments with three mice per group. *p < 0.05, ***p < 0.005.
FIGURE 2. LPS-mediated suppression of CD8 T cell responses depends on the TLR4–MyD88 signaling axis in CD11c+ cells. (A) WT and TLR2/4 double KO mice received WT OT-I T cells and were immunized as indicated. Representative plots show CD8 T cells positive for GFP and Kb tetramer. (B) Means ± SEM of GFP–OT-I T cells as a percentage of total CD8 T cells from three independent mice per group. (C) WT OT-I T cells were transferred into WT, TRIF KO, and MyD88 KO mice and immunized with OVA or OVA mixed with LPS. Representative plots show CD8 T cells positive for GFP and Kb tetramer. (D) Means ± SEM of GFP–OT-I T cells as a percentage of total CD8 T cells from three independent mice per group. (E) After OT-I CD8 T cell transfer, WT and CD11c MyD88 Tg mice were immunized as indicated and cells were stained for CD8 and Kb tetramer, and representative plots show percentage of CD8 T cells that expressed GFP and stained positive for the tetramer. (F) Means ± SEM of GFP–OT-I T cells as a percentage of total CD8 T cells from three independent mice per group. All data above are representative of two independent experiments with three mice per group. *p < 0.05, **p < 0.01, ***p < 0.005.
Consistent with our results from immunization using OVA, mice infected with VSV-OVA together with LPS or BLP, respectively, showed a marked reduction in CD8 T cell responses (Fig. 3A–D, Supplemental Fig. 3A). We observed that coinfections of mice with VSV-OVA and live \textit{S. typhimurium} also led to similar suppression of CD8 T cell responses (Fig. 3E, 3F). Heat-killed \textit{S. typhimurium} when coinjected with VSV-OVA also behaved similar to LPS and led to significant dampening of CD8 T cell responses induced by the virus (Fig. 3E, 3F). These results support the notion that TLRs play a critical role in regulation of CD8 T cell responses in the event of viral infections in that activation of TLR2 or TLR4 by bacterial ligands can suppress antiviral CD8 immunity.

Several cytosolic and vacuolar bacterial pathogens also induce robust CD8 T cell responses, and we were interested in understanding whether TLR2 and TLR4 activation would dampen CD8 T cell responses induced by intracellular bacteria. We used LM-OVA and tested the ability of TLR2 and TLR4 ligands to sup-

**FIGURE 3.** Antiviral CD8 responses are compromised by activation of plasma membrane TLRs. (A) Mice that received OT-I CD8 T cells were infected using VSV-OVA or VSV-OVA mixed with different TLR ligands or live/heat killed \textit{S. typhimurium} (Sal/Sal-HK), and draining LNs and the spleen were harvested on day 7 postinfection to measure OT-I CD8 T cell expansion. (A, C, and E). Representative plots show CD8 T cells that express GFP and stain positive for K\textsuperscript{b}-SIINFEKL tetramer. (B, D, and F). Means ± SEM of GFP–OT-I T cells as a percentage of total CD8 T cells from five independent mice per group. The data are representative of three (A–D) or two independent experiments (E, F). *p < 0.05, **p < 0.01, ***p < 0.005.
press CD8 T cell responses. As expected, LM-OVA induced robust CD8 T cell responses both in the spleen and the LNs (Fig. 4A). However, both LPS and BLP significantly dampened CD8 T cell expansion induced by LM-OVA (Fig. 4A, 4B, Supplemental Fig. 3B). Mice were also coinfected with LM-OVA and heat-killed Salmonella, and consistent with the VSV-OVA experiments, heat-killed Salmonella compromised the CD8 T cell response induced by LM-OVA (Fig. 4C, 4D). Although the VSV and Listeria experiments phenocopy the results of soluble protein immunizations, it is possible that type I IFNs (IFN-β and IFN-α) induced by LPS alter the ability of these pathogens to replicate, lowering the pathogen load and thus affecting the magnitude of CD8 T cell responses. Importantly, however, note that BLP (Pam3CSK4), which does not induce type I IFN production (35), also reduces the magnitude of CD8 T cell responses induced by VSV-OVA (Supplemental Fig. 3C). To still consider the possibility that LPS-induced type I IFNs could be affecting bacterial replication, we measured Listeria load in the LNs at day 3 following injection and saw no difference in the bacterial burden irrespective of whether the mice received LPS (Supplemental Fig. 3D). We could not detect bacteria in the draining LNs at time points earlier than day 3. Furthermore, when we looked at bacterial burden at day 7 following infection, we saw that, consistent with lower CD8 T cell responses, the mice that received LPS had higher bacterial loads in the liver, LNs, and the spleen (SupplementalFig. 3E). The LM-OVA results are very intriguing because Listeria has natural ligands to activate TLR2. It has been observed in an earlier study that L. monocytogenes infection of TLR2 KO mice led to a greater CD8 T cell response when compared with WT mice (37). It is possible that Listeria evades detection by TLR2, thus allowing the immune system to mount robust CD8 T cell responses. Our data clearly establish the dominant ability of surface TLRs to inhibit the

FIGURE 4. TLR2 and TLR4 activation inhibit CD8 T cell responses against L. monocytogenes. After OT-I T cell transfer, mice were infected with LM-OVA or LM-OVA mixed with LPS, BLP, or heat-killed S. typhimurium (Sal-HK). (A and C) Representative plots show CD8 T cells from draining LNs and spleen that express GFP and stain positive for Kb tetramer. (B and D) Means ± SEM of GFP–OT-I T cells as a percentage of total CD8 T cells from three independent mice per group. **p < 0.01, ***p < 0.005, ****p < 0.001.
CD8 T cell response induced by both a live virus and a cytosolic bacterium.

**LPS-mediated suppression is independent of Tregs and CD4 T cells**

Tregs are known to play a critical role in regulating immune responses. Using different experimental systems, several studies have shown the ability of Tregs to suppress CD8 T cell responses (38, 39). Other studies have shown that IL-10 secreted by Ag-specific non-Treg CD4 T cells can also suppress CD8 T cell responses in a CD25- and Foxp3-independent manner (19, 26). Therefore, we tested whether there is a potential role for Tregs in LPS-mediated CD8 T cell suppression. We depleted Tregs in vivo using an anti-CD25 Ab and immunized these mice with OVA with or without different TLR ligands. Treg depletion led to a slight basal enhancement of CD8 T cell responses in all groups but did not abrogate the ability of LPS to limit expansion of CD8 T cells (Fig. 5A, 5B). The extent of LPS-mediated suppression was similar to the control group of mice that received rat IgG. These results support the idea that Tregs have a very limited role in LPS-mediated impairment of CD8 T cell priming and expansion. Next we assessed the role of total CD4 T cells in LPS-mediated suppression of CD8 T cell responses in vivo. CD4 T cells were depleted using the GK1.5 mAb, and these mice were immunized...
with OVA in the presence or absence of TLR ligands. CD4 depletion led to an overall enhancement of CD8 T cell expansion in all groups, including the mice that received OVA plus LPS. However, LPS inhibited the CD8 T cell response in a significant manner (Fig. 5C, 5D). Similar to Treg depletion, these results demonstrate that CD8 T cells have little to no influence on LPS-mediated suppression of CD8 T cell responses.

**Differential recruitment of myeloid cells by TLR4 activation**

Many studies have revealed a variety of ways in which different myeloid-derived cells exert modulatory effects on immune responses (40). The suppressive role of myeloid suppressors has been reported in both cancer and infection models (41–43). In hopes of clarifying how TLR ligation on APCs could alter CD8 T cell responses, we began with a phenotypic analysis of APCs at early time points after immunization. We analyzed cells from draining LNs at 1, 6, 12, 24, and 48 h after immunization to look at different population of cells recruited to the draining LNs. We found that OV A plus LPS–immunized mice show a significantly enhanced CD11b+Ly6CintLy6G+ population of cells (Supplemental Fig. 4A) compared with the OV A and OV A plus CpG–immunized groups. Several recent studies have reported that CD11b+Ly6G+ neutrophils influence Ag presentation to T lymphocytes (44–46). Because this was a major cell population that was different between LPS-immunized and other groups of mice, we tested the possibility that this population of cells could directly suppress the CD8 T cell responses. We depleted these cells using a Ly6G mAb (clone 1A8) and the mice were immunized with OV A with or without LPS. Depletion of the Ly6G population did not relieve the inhibitory effects of LPS on CD8 T cell responses (Supplemental Fig. 4B, 4C).

**LPS fails to inhibit CD8 responses induced by peptide immunization**

Although the enhanced recruitment of Ly6G+ cells by LPS immunization is interesting, our experiments above make it unlikely that LPS induces recruitment of any kind of specialized cells that could be directly suppressing CD8 T cell responses in trans. However, it is possible that LPS affects the ability of DCs to target Ags to the cross-presentation pathway. We wanted to understand whether we could abrogate the inhibitory effects of LPS on CD8 T cell expansion by immunizing with a peptide and thus bypassing the MHC class I presentation pathway. We immunized mice with SIINFEKL in IFA or SIINFEKL plus LPS mixed in IFA and measured CD8 responses 7 d after immunization. SIINFEKL mixed with IFA induced robust expansion of CD8 T cells in the draining LNs. However, unlike OVA protein or VSV-OVA– and LM-OVA–induced CD8 responses, LPS failed to inhibit SIINFEKL-induced CD8 T cell priming and expansion (Supplemental Fig. 4D, 4E). These data are a clear indication that the inhibitory effects of TLR2 and TLR4 activation are because of their effects on the ability of APCs to cross-present Ags on MHC class I.

**LPS alters the DC populations in the draining LNs and directly affects the ability of DCs to present Ag to CD8 T cells**

The experiments above strongly suggest the possibility that LPS could influence the directing and processing of cargo inside the cell. This could lead to compromised presentation of peptides on MHC class I, and our data suggest that we could bypass that influence by providing a processed peptide (Supplemental Fig. 4D, 4E). To further address the possibility that LPS could affect cross-presentation of peptides on MHC class I, we sorted cells that take up Ag in the draining LNs following immunization. We used OVA-AF647 and immunized mice with or without different TLR ligands. We observed that the cells that took up OVA under different conditions were all CD11c+ DCs. After 18–24 h, OVA-AF647+ cells (Fig. 6A) were sorted and were cocultured at titrating concentrations with sorted naive OT-I CD8 T cells. This experiment allowed us to directly investigate the ability of APCs from different groups of mice to prime OT-I CD8 T cells. We found that the sorted APCs from OVA-AF647 alone or the OVA-AF647 plus CpG–immunized group induced OT-I proliferation, whereas APCs from the OVA-AF647 plus LPS–immunized group were defective in their ability to induce OT-I proliferation (Fig. 6B, 6C). Additionally, sorted APCs from the OVA plus CpG plus LPS–immunized mice were also defective in priming OT-I T cells compared with APCs from the OVA plus CpG–immunized group, again highlighting the dominant effect of TLR4 over TLR9 in the regulation of CD8 T cell priming (Fig. 6B, 6C). There was no major difference in the levels of MHC class I molecules expressed by APCs from any of these groups (Supplemental Fig. 4F). It is also possible that APCs from LPS-immunized mice secrete soluble factors that inhibit CD8 T cell priming. The role of soluble factors secreted by APCs upon TLR engagement have been implicated in CD8 T cell priming, and specifically LPS-induced IL-10 production has been linked to the inhibition of CD8 T cell responses (26, 47). To address this possibility, we mixed sorted APCs from OVA-AF647 plus CpG and OVA-AF647 plus LPS in a 1:1 ratio and observed no inhibition of CD8 T cell priming when compared with the OVA-AF647 plus CpG group (Fig. 6D). Rather, the mixing of APCs induced greater OT-I proliferation in an additive manner.

We wanted to explore the possibility that LPS induces differential recruitment of DC subpopulations in the draining LNs. For example, it is well known that lymphoid DCs are able to induce cross-presentation of exogenous Ags and induce CD8 T cell priming against extracellular Ags (48–50). Alternatively, myeloid DCs are important for CD4 T cell priming. We performed further characterization of DCs that had taken up OVA-AF647 and observed that the mice that received LPS had a lowered representation of lymphoid DCs in the total DC pool (Fig. 6E). These results suggest that LPS-mediated suppression is not due to soluble factors secreted by different APCs but rather because of its effect on differential recruitment of lymphoid versus myeloid DCs.

**Discussion**

It is now well established that stimulation of TLRs and/or other PRRs in DCs is important for activation and differentiation of naive CD4 T cells (13, 51). CD4 T cells play an important role in different facets of adaptive immunity. They are important to secrete effector cytokines that mobilize macrophages and neutrophils to the site of infection, induce activation and differentiation of B cells, and assist in the generation and functioning of CD8 memory T cells. Although it is therefore important for the immune system to induce CD4 T cell responses to all pathogens, irrespective of whether they are extracellular or intracellular, the usefulness of CD8 T cell responses for extracellular pathogens is less clear.

In the present study, we examined the effects of different TLRs on the regulation of CD8 T cell responses. Although all TLR ligands induced cross-presentation of soluble Ags in vitro, our in vivo experiments revealed an important biological understanding on how different TLRs regulate CD8 T cell priming in the lymphoid organs. In particular, we discovered that plasma membrane TLRs and endosomal TLRs have diametrically opposite effects on CD8 T cell priming and expansion in vivo. In agreement with previous findings, we observed enhanced CD8 T cell responses to TLR3 and TLR9 ligands, supporting the notion that these endosomal TLR ligands can be used as adjuvants to help induce CD8 T cell responses (16–18, 52). There are several reports on the effects of the TLR4...
ligand LPS on CD8 T cell responses (15, 25, 26, 47, 52–54). Many reports indicate that signaling through TLR4 inhibits CD8 priming (15, 52, 53) whereas other studies indicate that LPS enhances CD8 activation (54, 55). We observe a very robust re-

FIGURE 6. TLR4 activation in vivo affects the ability of APCs to present Ag to CD8 T cells and induces differential recruitment of lymphoid and myeloid DCs. Mice were injected with OVA or OVA-AF647 with or without different TLR ligands. (A) Cells that take up OVA can be identified in the draining LNs by flow cytometry. (B and C) OVA+ cells from the draining LNs of different groups of mice were sorted and incubated with OT-I CD8 T cells and proliferation was measured at the end of 72 h by a [3H]thymidine incorporation assay. (D) OVA+ APCs from draining LNs of OVA-primed group were mixed with the OVA+ APCs from the OVA plus LPS group and incubated with OT-I CD8 T cells, and proliferation of CD8 T cells was measured as described above. (E) Mice were immunized with OVA-AF647 with or without different TLR ligands and 24 h later cells from draining LNs were analyzed for OVA-AF647+ cells and further analyzed for expression of lymphoid DCs (CD11c+, CD8−, CD11b−) and myeloid DC markers (CD11c+, CD11b+, CD8−). Representative plot shows staining for CD11b and CD8 on CD11c+ cells. (F) Ratio of lymphoid DCs to myeloid DCs from several independent mice following immunization and staining as described in (E). *p < 0.05, **p < 0.01, ***p < 0.005.
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assessing the importance of TLR2 in inducing anti-
duction of innate and adaptive immunity against
infection. An earlier study in fact observed higher CD8 T cell
exploit this effect to reduce CD8 T cell responses during an active
CD8 T cell responses induced by
behaved similar to LPS and BLP and dramatically suppressed the
limited to soluble Ags. Additionally, heat-killed
ment of CD8 T cell responses, these responses were still suppressed
although CD4 T cell and Treg cell depletion led to basal enhance-
contamination of NOD or other cytosolic ligands. Additionally,
clearly establish that the suppression by LPS is not due to possible
priming mediated by TLR3 and TLR9. Our experiments involv-
TLRs had a dominant effect of inhibiting enhanced CD8 T cell
priming mediated by TLR3 and TLR9. Our experiments involv-
ing transfer of OT-I T cells into TLR2/TLR4 double KO mice
clearly establish that the suppression by LPS is not due to possible
contamination of NOD or other cytosolic ligands. Additionally,
although CD4 T cell and Treg cell depletion led to basal enhance-
ment of CD8 T cell responses, these responses were still suppressed
by LPS, suggesting that there are additional mechanisms that lead to
LPS- and BLP-induced suppression of CD8 T cell priming.

Our experiments using VSV and L. monocytogenes also establish
that suppression of CD8 T cell priming by LPS and BLP is not
limited to soluble Ags. Additionally, heat-killed S. typhimurium
behaved similar to LPS and BLP and dramatically suppressed
the CD8 T cell responses induced by Listeria and VSV. The Listeria
data are especially intriguing because the pathogen naturally con-
tains TLR2 ligands and suggests the possibility that Listeria could
exploit this effect to reduce CD8 T cell responses during an active
infection. An earlier study in fact observed higher CD8 T cell
responses in TLR2-deficient mice following Listeria infection
(37). It is also possible that TLR2 plays differential roles in in-
duction of innate and adaptive immunity against Listeria. Dif-
ferent experimental systems could lead to different results in
assessing the importance of TLR2 in inducing anti-Listeria im-
munity. It is clear that TLR2-mediated detection of Listeria is
important for host protection, and also other aspects of anti-Lis-
teria immune responses such as CD4 responses are unlikely to be
hampered by TLR2 signaling. Our studies only reveal previously
unappreciated effects of enhanced TLR2 ligation on the outcome
of anti-Listeria CD8 responses. We observe that only additional
activation of TLR2 by BLP during infection with Listeria led to
a dramatic reduction of CD8 T cell responses. These experiments
have important implications for the outcome of adaptive immu-
nity during combined multiple infections and suggest that anti-
viral or antibacterial CD8, but not CD4, responses could be
comprised under such circumstances. CD8 T cell responses can
be highly destructive and can induce severe pathology by killing
infected or peptide-loaded cells, and suppression of CD8 responses
could also allow induction of CD4 immune responses against rele-
vant pathogens that need CD4 T cells for clearance and protection.

It is possible that type I IFNs (IFN-β and IFN-α4) induced by
LPS alter the ability of Listeria or VSV to replicate, lowering their
load and thus affecting the magnitude of CD8 T cell responses.
However, we show that both TLR4 and TLR2 ligands inhibit OVA
(as well as L. monocytogenes and VSV)-induced CD8 responses.
Although vaccinia virus via TLR2 can induce type I IFNs, bac-
terial ligands such as Pam3CSK4 do not induce type I IFNs (35),
and in agreement with these findings we do not see any type I IFN
production by DCs stimulated with Pam3CSK4 (data not shown).
Moreover, CPG that induces type I IFNs does not inhibit CD8
responses induced by VSV. Thus, it is highly unlikely that type I
IFN production by LPS causes reduced viral load leading to re-
duced CD8 T cell priming. The VSV data are in support of the
data with OVA and Listeria and strengthen the overall argument
that TLR2 and TLR4 signaling have a dominant effect of down-
regulating CD8 T cell responses generated by both soluble pro-
teins and infectious agents. Additionally, a clear common theme
that emerges from our experiments is that the location of the TLR
at the time of signaling is an important determinant of the outcome
of CD8 T cell responses. For example, the plasma membrane
TLRs, TLR2 and TLR4, do not induce CD8 T cell priming in vivo,
whereas the endosomal TLRs, TLR3 and TLR9, enhance CD8 T
cell priming and expansion. It is known that TLR4 can signal
from both plasma membrane and endosomes in a MyD88- and
TRIF-dependent manner, respectively (59), and it is clear from our
data that TLR4-mediated suppression of CD8 T cell responses is
dependent on MyD88 and not the TRIF pathway of signaling.
Also, although endosomal TLRs traffic through different routes
before localization to the endosome (60), the final location of the
endosomal TLRs at the time of signaling seems to be important
to enhance CD8 T cell expansion. It is also very interesting that
although TLR9, TLR4, and TLR2 all use MyD88 as a signaling
adapter, the suppression mediated by TLR4 is dependent on
MyD88, suggesting that the biological outcomes of MyD88 sig-
naling downstream of different TLRs are vastly different. Impor-
tantly, note that the TRIF/IRF3 pathway of signaling is critical for
basal cross-presentation of Ags to CD8 T cells. The defects in
CD8 T cell priming in TRIF- and IRF3-deficient mice were, how-
ever, overcome by activation of the MyD88 signaling pathway
downstream of TLR9 (data not shown).

There could be several mechanisms by which TLR2 and TLR4
could be inhibiting activation of CD8 T cells. An attractive hy-
thesis is that plasma membrane TLRs could induce recruitment of
cells that suppress priming of CD8 T cells. Interestingly, we
found that LPS injection led to recruitment of a Ly6G+ popu-
lation of cells, but depletion experiments suggested that this popu-
lation was not responsible for the inhibition of CD8 T cell priming.
There are however several reports demonstrating that myeloid-
derived cells play a prominent role in immune suppression to
chronic viral infections and different cancers (41, 42). The im-
portance of recruitment of this population of cells for adaptive
immunity needs to be further investigated. A second possibility
is that activation of TLR2 and TLR4 altered the DC/T cell interac-
tion by interfering with levels of MHC class I. We observed no
differences in the level of MHC class I expressed on DCs in the
draining LNs, and when mice were immunized with peptide in-
stead of whole protein, LPS was no longer able to inhibit CD8
T cell priming.
Our final set of experiments provides compelling evidence that activation of TLR4 and TLR2 in vivo affects the ability of DCs to prime CD8 T cells. DCs from mice that received LPS were clearly deficient in their ability to activate OT-I CD8 T cells in vitro. Given that there is no alteration in levels of MHC class I and that peptide-induced CD8 T cell expansion is not inhibited by LPS, the reduced ability of DCs from either LPS-inmunized or LPS plus CpG-immunized mice could be because of alteration in lymphoid to myeloid DC ratios in the draining LNs. The lower number of lymphoid DCs in the draining LNs can lead to reduced priming of CD8 T cells. Our in vitro DC mixing experiments also provide important evidence that there is no active inhibition of CD8 T cells, either by a contact-dependent manner or by secretion of soluble suppressive factors by DCs from LPS-injected mice. The experiment in chimeric mice that express MyD88 only in CD11c+ DCs and macrophages also supports the notion that TLR4–MyD88 signaling in a cell-intrinsic fashion could regulate handling of the cargo inside the cells. Alternatively, the TLR9–MyD88 signaling axis promotes cross-presentation of Ags on MHC class I. The exact mechanism of how different DCs are recruited to the draining LNs needs further investigation. It also remains to be examined whether an individual DC makes distinct decisions of whether to promote or diminish targeting of Ags to the MHC class I pathway depending on the TLR that is activated. Given that neither TLR2 nor TLR4 activation in vitro hampers the ability of DCs to activate CD8 T cells, it is more likely that the specific composition of the DC populations at the time of CD8 T cell priming determines the outcome of CD8 T cell activation and expansion. Our work provides important insights on how plasma membrane and endosomal TLRs influence CD8 T cell priming, and these results have important implications for choosing TLR adjuvants for vaccines as well as for understanding how antiviral immunity could be hampered by bacterial coinfections.

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Disclosures

The authors have no financial conflicts of interest.

References


**Figure S1.** All TLR ligands induce cross-priming in vitro. (A) Splenic DCs were incubated with purified CFSE labeled OT-I CD8 T cells in the presence of OVA (20 μg/ml) with or without different TLR ligands (BLP, 1 μg/ml; LPS, 100 ng/ml; CpG, 1 μM; Poly IC, 1 μg/ml). Flow plots show CFSE dilution and forward scatter of live CD8 T cells after 3 days of culture. Data are representative of five independent experiments. **TLR4 activation in vivo suppresses CD8 but not CD4 T cell expansion.** (B-C) Mice (CD45.2) received a mixed transfer of GFP-OT-I CD8 T cells (1000 per mouse) and congenic (CD45.1) OT-II CD4 T cells (100,000 per mouse) and were immunized with OVA or OVA mixed with LPS in IFA. Seven days post immunization draining lymph nodes were stained for CD45.1, CD4 and CD8 markers. (B) Flow plots show percentage of CD4+ T cells (upper panel) that stain positive for CD45.1 or CD8+ T cells that express GFP (lower panel). (C) Mean ± SEM of percentage OT-II (upper panel) or OT-I (lower panel) T cells of total CD4 or CD8 T cell respectively. Data are representative of three independent experiments. **Kinetics of CD8 T cell expansion at different time points after OVA with or without LPS immunization.** (D) Mice that received GFP-OT-I CD8 T cells were immunized with OVA or OVA+LPS in IFA and cells from the draining lymph nodes were stained using Kb-SIINFEKL tetramer at indicated time points following immunization. Representative plot of three independent experiments show percentage of CD8 T cells that express GFP and stained positive for tetramer. *, P < 0.05; **, P < 0.01.
Figure S2. Basal cross-presentation of soluble antigens depends on the TRIF-IRF3 signaling pathway. (A) WT, TRIF KO and IRF3 KO mice that received GFP-OT-I T cells were immunized with OVA or OVA with LPS and cells from the draining lymph node were stained using Kb-SIINFEKL tetramer. Representative plots show CD8 T cells that express GFP and stain positive for the tetramer. Inhibition of CD8 T cell priming by LPS depends on MyD88 expression in DCs. (B) WT and CD11c-MyD88Tg mice were immunized as described above and cells from draining lymph nodes were stained using Kb-SIINFEKL tetramer. Representative flow plots show CD8 T cells that stain positive for the tetramer. Data are representative of three independent experiments.
Figure S3. Surface TLR ligands suppress endogenous CD8 T cell responses against VSV and Listeria monocytogenes.

Mice without OT-I T cell transfer were infected with either VSV-OVA (A) or LM-OVA (B) without or without BLP and LPS. Representative plots show cells in the draining lymph nodes that express CD8 and stain positive for K\textsuperscript{b}-SIINFEKL tetramer. **TLR9 activation does not suppress VSV induced CD8 T cell responses.** (C) Mice that received GFP-OT-I T cells were infected with VSV-OVA or VSV-OVA with different TLR ligands and 7 days after immunization, cells from draining lymph nodes were stained to measure the CD8 T cell expansion. **Quantification of bacterial burden at different time points following infection.** (D-E) Mice were infected with LM-OVA or LM-OVA with LPS. Three days (C) and seven days (D) of post-infection bacterial burden was quantified by plating on BE-agar streptomycin plates. Representative plots show LM CFU in indicated tissue.
Figure S4. Accumulation of a Ly6G+ population in the draining lymph nodes of LPS immunized mice does not cause CD8 T cell suppression and TLR4 activation does not suppress peptide induced CD8 T cell responses or alter the level of MHC Class I molecules on antigen presenting cells. (A) Draining lymph nodes of mice that were immunized with OVA or OVA+ LPS in IFA were stained at 12 hours following immunization. Flow plots show CD11c+ CD11b+ cells in the lymph nodes that express Ly6G and Ly6C. (B) Mice were given anti-Ly6G antibody (Clone 1A8) either once (1A8-1) or twice (1A8-2) as described to deplete Ly6G expressing cells and were then immunized as indicated after OT-I CD8 T cell transfer. Representative plots show CD8 T cells that express GFP and stain positive for the tetramer. (C) Mean ± SEM of percentage GFP-OT-I T cells of total CD8 T cell population. Data are representative of two independent experiments. *, P < 0.05; **, P < 0.01. (D-F) Mice that received OT-I CD8 T cells were immunized with SIINFEKL (1 µg/mouse) or SIINFEKL+ LPS emulsified in IFA and draining lymph nodes were harvested 7 days after immunization. (D) Representative plots show CD8 T cells that express GFP and stain positive for the tetramer. (E) Mean ± SEM of percentage GFP-OT-I T cells of total CD8 T cell population from 3 independent mice. Data are representative of five independent experiments. (F) Mice were injected with OVA-AF647 with or without TLR ligands and 24 hours later cells from draining lymph nodes were stained for expression of MHC class I molecules. OVA-AF647+ cells were analyzed for expression of MHC Class I (Kb). Data are representative of three independent experiments.